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The *C. elegans hunchback* Homolog, *hbl-1*, Controls Temporal Patterning and Is a Probable MicroRNA Target

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Summary

***hunchback* regulates the temporal identity of neuroblasts in *Drosophila.* Here we show that *hbl-1*, the *C. elegans hunchback* ortholog, also controls temporal patterning. Furthermore, *hbl-1* is a probable target of microRNA regulation through its 3′UTR. *hbl-1* loss-of-function causes the precocious expression of adult seam cell fates. This phenotype is similar to loss-of-function of *lin-41*, a known target of the *let-7* microRNA. Like *lin-41* mutations, *hbl-1* loss-of-function partially suppresses a *let-7* mutation. The *hbl-1* 3′UTR is both necessary and sufficient to downregulate a reporter gene during development, and the *let-7* and *lin-4* microRNAs are both required for HBL-1/GFP downregulation. Multiple elements in the *hbl-1* 3′UTR show complementarity to regulatory microRNAs, suggesting that microRNAs directly control *hbl-1*. MicroRNAs may likewise function to regulate *Drosophila hunchback* during temporal patterning of the nervous system.**

Introduction

Normal development requires that each cell in the developing organism adopt the correct positional and temporal identity. One class of temporal patterning genes belongs to the heterochronic pathway (Ambros and Horvitz, 1984; Banerjee and Slack, 2002). Mutations in heterochronic genes cause the temporal misexpression of cell fate programs, such that stage-specific programs are inappropriately skipped or reiterated, relative to wild-type. For example, loss-of-function mutations in the *C. elegans* heterochronic gene *lin-41* cause hypodermal (skin) seam cells to inappropriately adopt adult fates in the fourth larval stage; conversely, overexpression of *lin-41* causes the seam cells to reiterate larval fates at the adult stage (Slack et al., 2000). Thus, the *lin-41* gene encodes a key developmental switch that represses adult seam cell fates until their proper expression time. LIN-41 controls the time when the adult specification transcription factor LIN-29 is expressed. LIN-29 is expressed precociously in *lin-41* mutants, resulting in premature adoption of adult fates (Slack et al., 2000). Since *lin-29* mRNA is normally detected at early larval stages (Rougvie and Ambros, 1995) and the protein is not observed until the L4 stage (Bettinger et al., 1996), it is likely that *lin-29* is posttranscriptionally controlled by LIN-41.

Downregulation of *lin-41* during L4 is mediated by the *let-7* regulatory RNA (Reinhart et al., 2000; Slack et al., 2000). Normally, *let-7* inhibition of LIN-41 protein expression upregulates *lin-29* expression. *let-7* mutations cause retarded cell lineage transformations: *let-7* mutants reiterate larval stage cell fates within adults and delay the onset of LIN-29 expression in L4 (Reinhart et al., 2000). *let-7* encodes a phylogenetically conserved, 21-nucleotide (nt) RNA that is expressed during L3 and later stages (Pasquinelli et al., 2000; Reinhart et al., 2000). *let-7* RNA negatively regulates *lin-41* via several complementary sites in the *lin-41* mRNA 3′UTR (Reinhart et al., 2000; Slack et al., 2000). Similar motifs exist in *Drosophila* and zebrafish *lin-41* 3′UTRs (Pasquinelli et al., 2000).

Because *let-7* and another heterochronic gene, *lin-4*, are temporally regulated RNAs that control temporal patterning, they are referred to as small temporal RNAs (stRNAs) (Pasquinelli et al., 2000). These stRNAs are founding members of a larger class of similarly sized microRNAs (miRNAs) (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). miRNAs are genomically encoded, transcribed as hairpin-structured pre-miRNAs, and processed by the Dicer (DCR-1 in *C. elegans*) nuclease to generate their 20–24 nt mature form (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001). So far, only three bona fide animal miRNA targets are known, all residing in the heterochronic pathway: *lin-14* and *lin-28* are regulated by *lin-4* (Lee et al., 1993; Moss et al., 1997; Wightman et al., 1993), while *lin-41* is controlled by *let-7* (Reinhart et al., 2000; Slack et al., 2000).

Although the molecular function of LIN-41 is yet to be identified, we favor a role in translational control (Slack et al., 2000). Consistent with the role we propose, LIN-41 is a *r*ing finger *B* box *c*oiled *c*oil-*NHL* domain (RBCC-NHL)-containing protein (Slack and Ruvkun, 1998) and thus belongs to the same family of proteins as *Drosophila* Brain Tumor (Brat) (Sonoda and Wharton, 2001). Brat is a translational repressor of *hunchback* (*hb*) expression in the *Drosophila* embryo. *hb*, a key patterning gene required for correct anterior structure specification, is expressed in a spatial gradient with high Hunchback protein levels in the embryonic anterior and low levels in the posterior (Irish et al., 1989). A complex of Nanos/

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Pumilio/Brat proteins binds to the *hb* mRNA 3′UTR and prevents Hb protein accumulation in the posterior through an unknown mechanism.

Here, we demonstrate that *C. elegans hbl-1* (*h*unch-*b*ack-*l*ike) (Fay et al., 1999), the ortholog to *Drosophila hb*, regulates temporal patterning. Temporal regulation of *hbl-1* expression in hypodermal and specific neuronal cells is partially mediated by its 3′UTR, which contains sites complementary to the *let-7* (and family), *lin-4*, and *mir-69* miRNAs. Regulation of *hbl-1* expression in ventral nerve cord neurons depends on *let-7* and *lin-4* miRNAs. Recently, *Drosophila hb* and *Krüppel* (*Kr*), long known for their embryonic spatial patterning roles, have been shown to function in temporal patterning as well (Isshiki et al., 2001). We posit that spatial and temporal patterning programs may share conserved factors and regulatory mechanisms utilizing miRNAs to pattern developing organisms.

Results

The Heterochronic Mutation *mg285* Is a Deletion in the *hbl-1* Gene

We identified a single, recessive allele of *hbl-1*, *mg285*, in a forward genetic screen for heterochronic mutants. We mapped *mg285* to a small interval on LGX near *lon-2*. Sequencing of the exons and exon-intron boundaries from *mg285* revealed a small deletion in the 5′ region of the *hbl-1* gene (Figures 1A and 1B), indicating that *mg285* is an *hbl-1* allele. In addition, RNA interference (RNAi) by feeding of *hbl-1* double-stranded (ds) RNA leads to a phenotype similar to *hbl-1(mg285)* (see below). Thus, *mg285* probably disrupts *hbl-1* and not another gene. *hbl-1(mg285)* deletes 301 base pairs in the 5′ region of *hbl-1*, causing a frameshift predicted to truncate HBL-1 protein in the third exon (Figure 1B). If there is only one *hbl-1*splice form, as reported (Fay et al., 1999), then *hbl-1(mg285)* is likely to be a null allele. However, RNAi by injection of *hbl-1* dsRNA gives a stronger phenotype than *hbl-1(mg285)*, including embryonic and larval lethality (Fay et al., 1999). Thus, we believe that *hbl-1(mg285)* is not a null allele. We suspect that a rare, alternatively spliced *hbl-1* variant exists that splices around the deletion and partially rescues the mutation or that there is downstream translation initiation producing a protein with partial activity. *hbl-1* was originally identified (Fay et al., 1999) by its homology to *Drosophila hb*, a transcription factor well known for its role in early *Drosophila* embryonic patterning along the anterior-posterior axis (Struhl et al., 1992).

hbl-1 Mutants Display a Heterochronic Defect

The *hbl-1(mg285)* mutation causes precocious expression of adult-specific hypodermal cell fates (Table 1; Figure 2). In wild-type animals, the lateral hypodermal seam cells divide with a stem cell-like pattern during the L1, L2, L3, and L4 stages before exiting the cell cycle, terminally differentiating and fusing together around the time of the L4 molt (Sulston and Horvitz, 1977), an event termed the larval to adult (L/A) switch. After fusion, these cells secrete a cuticular structure known as lateral alae. We examined the postembryonic development of the seam cells (V cells and descendants) in *hbl-1(mg285)* mutants. Seam cell development in hermaphrodites proceeded normally until the L3 molt, at which point most of the seam cells precociously underwent the L/A switch (number of animals scored, late L1, n = 38; late L2, n = 87; early L3, n = 97; early L4, n = 112). Eighty-nine percent of *hbl-1(mg285)* animals (n = 112) exhibited complete precocious seam cell fusion in the early L4 stage (Figure 2B), compared with 1% for wild-type animals (n = 73) (Figure 2A). A similar precocious heterochronic defect was observed when wild-type animals were subjected to postembryonic RNAi of *hbl-1* (Table 1). *hbl-1(mg285)* and *hbl-1(RNAi)* animals also displayed precocious alae formation at the early L4 stage (Table 1; Figure 2E). Thus, *hbl-1* is required to prevent adult seam cell fates from occurring before the adult stage.

In addition to functioning during early larval stages, *hbl-1* may also play a secondary, opposite role during the L4 or adult stage. Twenty-nine percent of *hbl-1(mg285)* young adult animals (n = 55) contained one or more breaks in the adult seam, compared with 3% of wild-type adults (n = 34). Similar results were obtained by growing wild-type animals on *hbl-1(RNAi)*, where 28% of young adults (n = 32) displayed breaks, compared with 0% (n = 24) of animals fed on empty vector. Ninety-five percent of *hbl-1(mg285)* adults (n = 55) also showed additional seam cell nuclei at the adult stage (up to 25 nuclei; average number, 20; Figure 2F), compared with 18% of wild-type animals (n = 34) (up to 18 nuclei; average number, 16). Similarly, 31% (n = 32) of wild-type animals fed on *hbl-1(RNAi)* displayed additional nuclei (up to 23 nuclei). These data suggest that *hbl-1* knockdown caused seam nuclei to divide inappropriately at the adult stage. In the analysis below, we focused on the earlier larval role for *hbl-1*.

hbl-1(mg285) Does Not Affect the Timing of *let-7* RNA Expression

One possible explanation of the *hbl-1* phenotypes is that *hbl-1* is upstream of *let-7* and regulates *let-7* expression. However, when we examined the *let-7* RNA temporal expression profile in wild-type and *hbl-1(mg285)* animals, we found no appreciable difference (Figure 1D). Thus, the precocious phenotypes of *hbl-1(mg285*) are not due to precocious *let-7* expression, nor are its retarded phenotypes due to retarded *let-7* expression. Therefore, *hbl-1* does not appear to act upstream of *let-7*. Similarly, a mutation in *lin-41*, a known downstream target of *let-7* RNA (Reinhart et al., 2000), does not affect the time of *let-7* expression during the late L3 stage (Johnson et al., 2003).

hbl-1 Negatively Regulates *lin-29*

LIN-29, a zinc finger transcription factor, normally appears at the L4 stage in hypodermal cells (Bettinger et al., 1996) and is necessary for terminal differentiation of these cells in the adult; in *lin-29* mutants, cells reiterate larval fates and continue to divide (Ambros and Horvitz, 1984). In many precocious heterochronic mutants, LIN-29 is prematurely expressed (Bettinger et al., 1996), and *lin-29* is epistatic to these precocious heterochronic mutations. We found that *hbl-1(RNAi); lin-29(n546)* double-knockdown animals do not display a precocious defect but, instead, look like a *lin-29* single mutant (Table

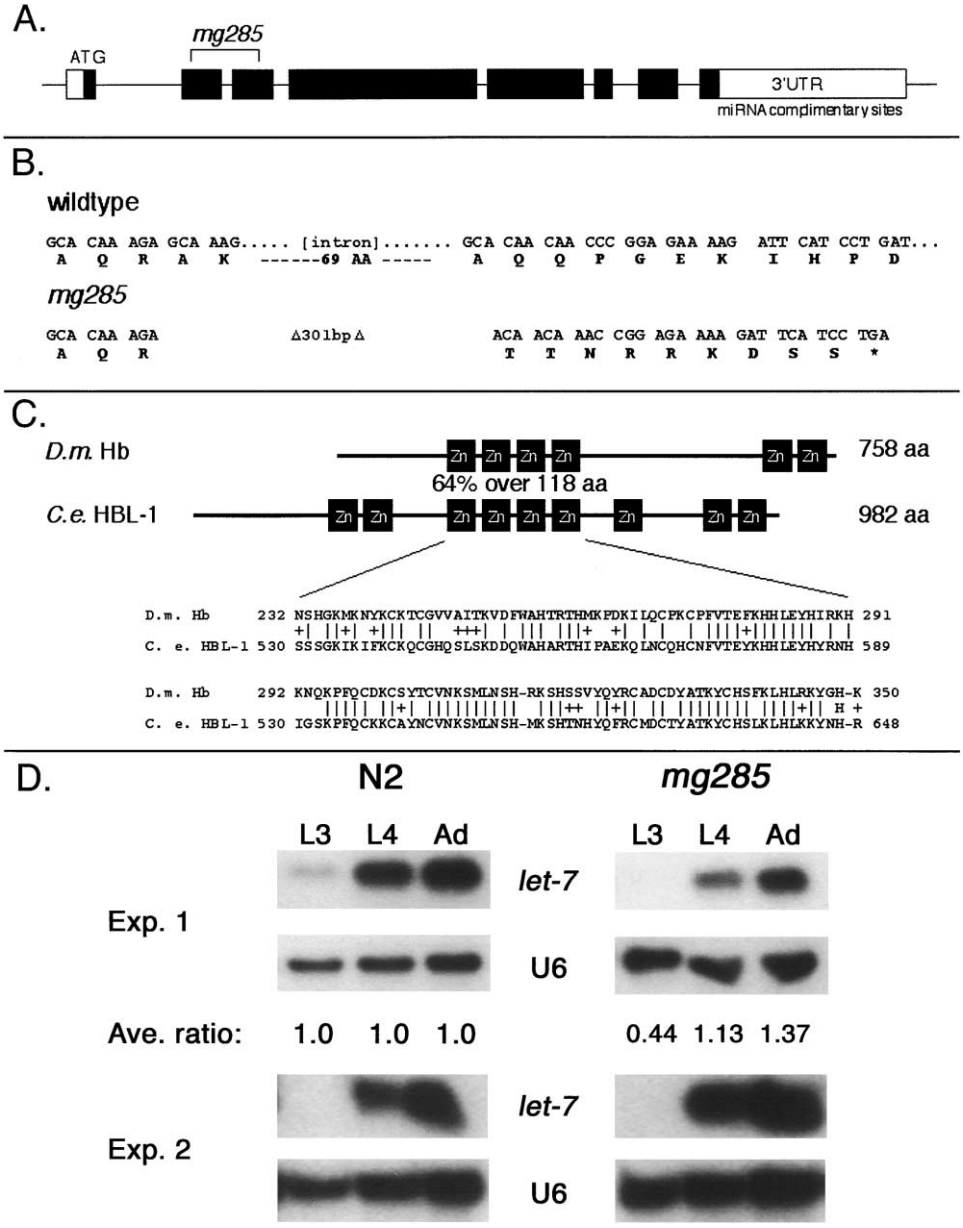

Figure 1. The *hbl-1* Gene

(A) The intron/exon structure of *hbl-1*. The relative position of the *mg285* allele is shown: a 301 bp deletion from position 18765 to position 19066 (numbering for cosmid F13D11, accession number U40939).

(B) In the *hbl-1(mg285)* mutant, intron 2 plus 69 codons from exons 2 and 3 are deleted, leading to a frameshift predicted to terminate in a premature stop codon, compared with the wild-type sequence. *mg285* also contains a C to A point mutation, 8 nt 3′ to the deletion endpoint.

(C) HBL-1 is a close relative of Hb (accession numbers AF097737 [*hbl-1*] and Y00274 [*hb*]). The amino acid sequence of the indicated zinc finger region of *C. elegans* (C.e.) HBL-1 is shown in an alignment with *D. melanogaster* (D.m.) Hb.

(D) *hbl-1(mg285)* does not affect the timing of *let-7* RNA expression. Northern blots prepared from RNA isolated from wild-type or *hbl-1(mg285)* animals at different developmental stages were hybridized with *let-7* and U6 probes. Two independent experiments reveal the same trend. Numbers represent the ratio of RNA in the *hbl-1* mutant versus the wild-type strain, normalized to the U6 loading control and averaged over the two experiments.

1). Thus, the *hbl-1(RNAi)* defect required a wild-type copy of *lin-29*. Since *lin-29(n546)* is a null allele (Rougvie and Ambros, 1995), this suggests that *hbl-1* negatively regulates *lin-29* in the hypodermis and, by extension, controls the L/A switch.

hbl-1 RNAi Enhances a *lin-41(ma104)* Allele

lin-41 null animals display a partially penetrant precocious defect, suggesting that other factors also control the L/A switch. Since both *lin-41* and *hbl-1* act through *lin-29* to negatively regulate the L/A switch, we tested

Table 1. *hbl-1* Interacts with *lin-41* and *lin-29* to Control the Temporal Identity of Seam Cells

Strain[a]	Percentage of Animals with Any Fused Seam Cells in Early L4 (n)	Percentage of Animals with Any Alae in Early L4 (n)
Wild-type	10 (73)	0 (73)
hbl-1(mg285)	100 (112)	51 (112)
Wild-type; *empty vector(RNAi)*[a]	0 (32)	0 (32)
Wild-type; *hbl-1(RNAi)*[a]	100 (43)	81 (43)
	Percentage of Animals with Complete Alae in Early L4 (n)	
Wild-type; *empty vector(RNAi)*	0 (32)	
Wild-type; *lin-41(RNAi)*	0 (32)	
hbl-1(mg285); empty vector(RNAi)	10 (52)	
hbl-1(mg285); lin-41(RNAi)	20 (41)	
	Percentage of Animals with Complete Seam Cell Fusion in Early L4 (n)	
Wild-type; *empty vector(RNAi)*	0 (43)	
hbl-1(mg285); empty vector(RNAi)	83 (52)	
Wild-type; *hbl-1(RNAi)*	65 (43)	
lin-29(n546); empty vector(RNAi)	0 (10)	
lin-29(n546); hbl-1(RNAi)	0 (10)	

[a] For *hbl-1(RNAi)*, *lin-41(RNAi)*, and *empty vector(RNAi)*, the indicated animals were synchronized by hatching prepared eggs in the absence of food and allowing the L1 animals to grow on HT115 *E. coli*-expressing ds *hbl-1* RNA, ds *lin-41* RNA, or RNA from an empty vector, respectively. For the seam cell fusion experiments, animals carried *wIs79.*

whether *hbl-1* and *lin-41* can function together. We found that more seam cells precociously execute the L/A switch in animals where both *hbl-1* and *lin-41* activities are depleted by non-null mutations or RNAi, compared with single-knockdown animals (Table 1). In addition, while *hbl-1(RNAi)* animals (5%, n = 20) and *lin-41(ma104)* animals (8%, n = 38) rarely display any signs of precocious alae in the L3 stage (two stages earlier than in the wild-type), reduction of both *lin-41* and *hbl-1* function [*lin-41(ma104); hbl-1(RNAi)*] caused more animals to show any precocious L3 alae (55%, n = 86). Thus, knockdown of both *hbl-1* and *lin-41* function results in reciprocally enhanced penetrance and expression of each other's precocious phenotypes. This suggests that *lin-41* and *hbl-1* act partially redundantly in the same, or parallel, pathways to affect the L/A switch.

Expression of *hbl-1* Is Temporally Controlled

HBL-1/GFP is expressed strongly in hypodermal cells, including the embryonic seam cell precursors, and in neurons like those of the ventral nerve cord (VNC) during postembryonic stages (Fay et al., 1999). We reexamined HBL-1/GFP expression, focusing on hypodermal and

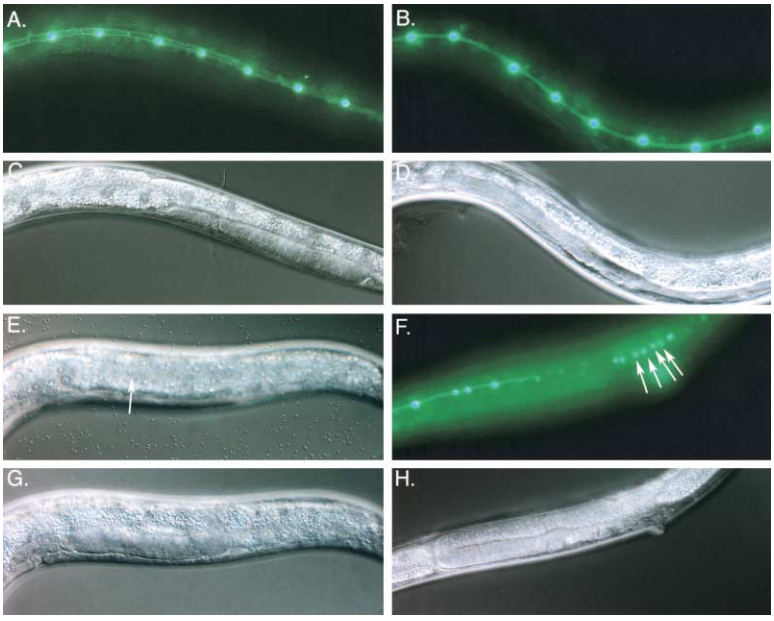

Figure 2. The Heterochronic Phenotypes of *hbl-1(mg285)*

Anterior is left; ventral is down; magnification, 400×.

(A) Wild-type L4 animal exhibiting normal unfused seam cells.

(B) *hbl-1(mg285)* L4 animal displaying precocious seam cell fusion.

(C and D) Nomarski images of the same animals as in (A) and (B), respectively, focused on the gonad to show developmental staging.

(E) Precocious alae formation in an *hbl-1(mg285)* L4 animal (arrow).

(F) *hbl-1(mg285)* adults form extra seam nuclei (arrows).

(G and H) Nomarski images of the gonads of the animals in (E) and (F), respectively.

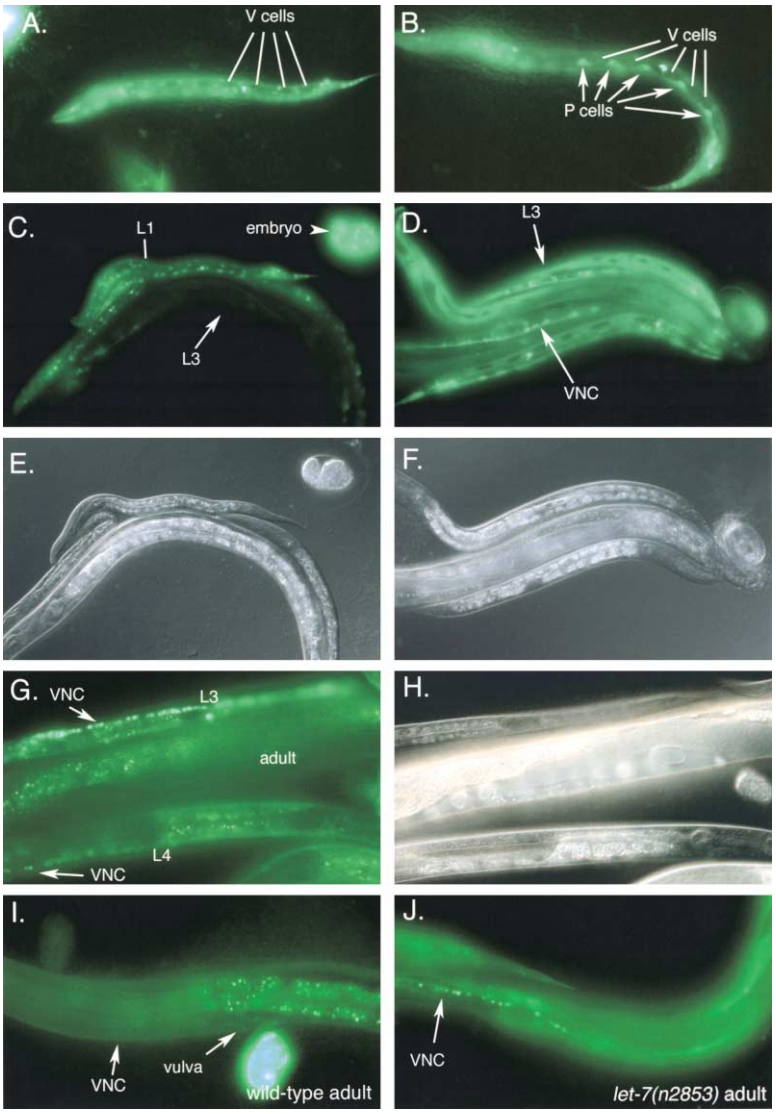

Figure 3. Postembryonic Expression Pattern of HBL-1/GFP in Wild-Type and *let-7* Animals

(A) An early L1 BW1932 animal showing HBL-1/GFP expression in hypodermal cells. Lateral seam cells (lines) are shadowed by more GFP expression in hyp7 (magnification, 400×). Nucleus of V6 (most-posterior line), green.

(B) Expression is largely absent from seam cells in a mid L1 BW1932 animal (lines) but is present in P cells (arrows) (magnification, 630×).

(C) HBL-1/GFP expression in BW1932 is most intense in embryos (arrowhead), decreases in L1s (line), and is virtually absent in the hypodermis of an L3 animal (arrow) (magnification, 400×).

(D) Strain BW1981 contains an integrated array similar to BW1932, except that the *hbl-1* 3′UTR is replaced by the *unc-54* 3′UTR and the nuclear localization signal on GFP is missing. HBL-1/GFP in the hypodermis of embryos, an L2 animal (bottom), an L3 animal (top), an L4 animal (middle) (except in seam cells, which are shown shadowed by the hyp7 expression), and in the VNC of the L4 (magnification, 400×). (C) and (D) were exposed for the same amount of time.

(E and F) Nomarski images of the same animals as in (C) and (D), respectively.

(G) HBL-1/GFP in BW1932 is most intense in the ventral nerve cord of L3 animals (an early L3 is shown), less intense in an L4 animal, and almost absent in an adult animal.

(H) A DIC image of the same animals as in (G).

(I) Wild-type BW1932 adult with barely detectable expression of HBL-1/GFP in the VNC, compared with a *let-7(n2853)* adult, shown in (J).

VNC expression at postembryonic *C. elegans* developmental stages. Strain BW1932 (Fay et al., 1999) contains an integrated array with the *hbl-1* promoter, the first 133 amino acids of HBL-1 fused to GFP, and the *hbl-1* 3′UTR. During the L1 stage, HBL-1/GFP expression was observed in the hypodermal syncitial cells (e.g., hyp7), in the ventral hypodermal cells (P cells), and weakly in the lateral hypodermal seam cells (H, V, and T cells) (Figures 3A and 3B). By the L2 stage, HBL-1/GFP was no longer expressed in the seam cells but was still observed in P cell descendants (Figure 3C) and weakly in the non-seam cell hypodermis. By the L3 stage, HBL-1/GFP was virtually absent in the hypodermis and Pn.p cell descendants, but was still highly expressed in the ventral nerve cord (generated from Pn.a cells) (100%, n = 40) and other unidentified neurons (Figures 3C and 3G). Early L4 animals express high HBL-1/GFP levels in the VNC (76%, n = 26), while late L4 and adult animals express HBL-1/GFP very weakly in the VNC (100%, n = 40) (Figure 3G). In some adult VNCs, expression is undetectable (Figure 3I). As judged by this HBL-1/GFP fusion, HBL-1 expression is downregulated during the course of postembryonic development, with highest expression in L1 animals and lowest expression in adults.

Posttranscriptional Control of *hbl-1* via 3′UTR Sequences

In many tissues, temporal downregulation of HBL-1/GFP during postembryonic development depended on sequences in the *hbl-1* 3′UTR, since an integrated *hbl-1::gfp* fusion gene with a heterologous 3′UTR from *unc-54* in strain BW1891 failed to display temporal downregulation in the non-seam cell hypodermis, in the VNC, and in other unidentified parts of nervous system (Figure 3D).

The *hbl-1* 3′UTR was sufficient to downregulate a heterologous reporter gene. When the *hbl-1* 3′UTR was placed 3′ to the *Escherichia coli lacZ* gene and driven by the hypodermally expressed *col-10* promoter (*zaEx6*), this reporter was temporally regulated; β-galactosidase activity was observed in 35% of L1s (n = 311), 43% of L2s (n = 76), 24% of L3s (n = 72), 5% of L4s (n = 85), and 2% of young adults (n = 95). The high frequency

of reporter gene expression in early larval animals, with decreased frequency during late larval stages, broadly mimics HBL-1/GFP hypodermal expression observed in vivo. Late stage downregulation of the *col-10::lacZ* fusion gene was dependent on the *hbl-1* 3′UTR because a control fusion gene bearing the 3′UTR of the nonheterochronic *unc-54* gene was expressed at all stages, including the adult stage (data not shown; Wightman et al., 1993).

By contrast, HBL-1/GFP downregulation in seam cells during the L1 stage is not 3′UTR dependent; downregulation was still observed in the fusion strain BW1891 (Figure 3D). Thus, *hbl-1* downregulation in the seam cells during the L1 stage is likely to be transcriptionally, rather than posttranscriptionally, regulated.

The *hbl-1* 3′UTR Contains Multiple Sequences Complementary to miRNAs, Including *let-7* and *lin-4*

let-7 and *lin-4* encode small, temporally expressed, regulatory RNAs that negatively regulate the expression of multiple heterochronic genes (Lee et al., 1993; Reinhart et al., 2000). Known genes that are regulated by the miRNAs contain sequences complementary to both *let-7* and *lin-4* in their 3′UTRs (Lee et al., 1993; Moss et al., 1997; Reinhart et al., 2000; Slack et al., 2000; Wightman et al., 1993). We identified eight potential *let-7* complementary sites (LCSs) and two potential *lin-4* complementary elements (LCEs) in the *hbl-1* 3′UTR (Figure 4). These sites are predicted to form RNA::RNA duplexes with the miRNAs (Figures 5A and 5B).

The literature reports four genes in the *C. elegans* genome that, like *let-7*, encode miRNA gene products whose expression is temporally regulated. Three of these miRNAs are likely to be *let-7* homologs, on the basis of their sequence similarity and expression profile (Lau et al., 2001). Therefore, while the LCS sites were originally identified by their ability to base pair with *let-7* RNA, it is possible that they may represent complementary sites for one of the *let-7* homologs. Consistent with this notion, we found that one of the *hbl-1* LCS sites (LCS 3) is more complementary to *mir-84*, a newly discovered miRNA that is similar to *let-7* in sequence and in its temporal expression (Lau et al., 2001). In addition to these three *let-7* homologs, Lau et al. (2001) have described one other temporally regulated miRNA, *mir-69*, whose sequence is unrelated to *let-7* RNA. We identified two sites in the *hbl-1* 3′UTR with the potential to base pair *mir-69* (Figures 4A, 4B, and 5C).

The recently sequenced genome of the closely related nematode, *C. briggsae*, contains a predicted *hbl-1* gene that is 81% identical at the amino acid level to *C. elegans* HBL-1 over the 337 amino acids across the zinc finger region. Most of the miRNA complementary sites in the *C. elegans hbl-1* 3′UTR are also conserved in the *C. briggsae hbl-1* 3′UTR, supporting that they are functionally relevant (Figure 4B).

hbl-1 Genetically Interacts with *let-7*

Consistent with our observations that *hbl-1* is not involved in *let-7* temporal regulation and that the *hbl-1* 3′UTR contains multiple conserved *let-7* RNA binding sites, we predicted that *hbl-1* is a negatively regulated target of *let-7*. If this were the case, then the temperature-sensitive lethal and retarded phenotypes caused by a *let- 7(n2853)* mutation (Reinhart et al., 2000) could be due to excessive *hbl-1* signaling, a defect that should be suppressed by loss of *hbl-1* function. We observed that the *let-7* lethal phenotype was indeed suppressed by *hbl-1(RNAi)*; 2% of *let-7(n2853)* mutants (n = 174) survived to adulthood at the nonpermissive temperature (20°C) when fed the empty vector control, but 93% (n = 196) of *let-7(n2853)* animals survived when fed *hbl-1(RNAi)*. Furthermore, 21% of *let-7(n2853)* animals fed *hbl-1(RNAi)* (n = 14) at the nonpermissive temperature displayed complete alae at the adult stage, compared with 0% (n = 12) of animals fed the empty vector RNAi. Thus, the retarded heterochronic phenotype of *let-7* was also partially suppressed by knockdown of *hbl-1* function.

let-7 and *lin-4* Regulate *hbl-1* Expression in the VNC

let-7 RNA is expressed predominantly in the L4 and adult stages (Pasquinelli et al., 2000; Reinhart et al., 2000). HBL-1/GFP expression in the VNC is downregulated during the L4 and adult stages by a 3′UTR-dependent mechanism. The similar timing of these two events suggested that *let-7* might be involved in downregulation of *hbl-1* in the VNC. Indeed, we found that, while 45% (n = 33) of *let-7(n2853)* adults expressed intense HBL-1/GFP in the VNC, only 4% (n = 45) of wild-type animals did the same (Figures 3I and 3J). *lin-4* RNA is also present in the L4 stage. We observed intense HBL-1/GFP expression in the VNC of 100% (n = 30) of *lin-4(e912)* adult animals (data not shown). Thus, both wild-type *let-7* and *lin-4* RNAs are required for proper *hbl-1* downregulation in the VNC.

However, *let-7(n2853)* had no detectable effect on proper downregulation of HBL-1/GFP expression in the hypodermis (data not shown). This is consistent with our observation that HBL-1/GFP is absent from the hypodermis by the time *let-7* RNA begins to be expressed during the L3 stage. We observed similar results with a hypodermally expressed reporter gene (*col-10::lacZ::hbl-1* 3′UTR), whose temporal downregulation was complete by the end of the L3 stage. *let-7* RNA was not required to downregulate the reporter (data not shown).

On the other hand, *lin-4* RNA is present at the right time to participate in HBL-1/GFP downregulation during the L2 stage in the hypodermis and Pn.p cell descendants. *lin-4* RNA is initially expressed toward the end of the L1 stage (Feinbaum and Ambros, 1999) and downregulates its targets *lin-14* and *lin-28* in the hypodermis. However, we did not observe an effect on HBL-1/GFP hypodermal expression in a *lin-4(e912)* null mutant background (data not shown). Since HBL-1/GFP downregulation during this stage requires the *hbl-1* 3′UTR, it is likely that other genes, e.g., other miRNAs, perform this function. Our results implicate both *let-7* and *lin-4* RNAs in the selective HBL-1/GFP temporal downregulation in the VNC, but not in the hypodermis.

Discussion

MicroRNAs are a new class of regulatory, noncoding RNAs. Over 100 miRNAs have been described from diverse animals (Lagos-Quintana et al., 2001, 2002; Lau

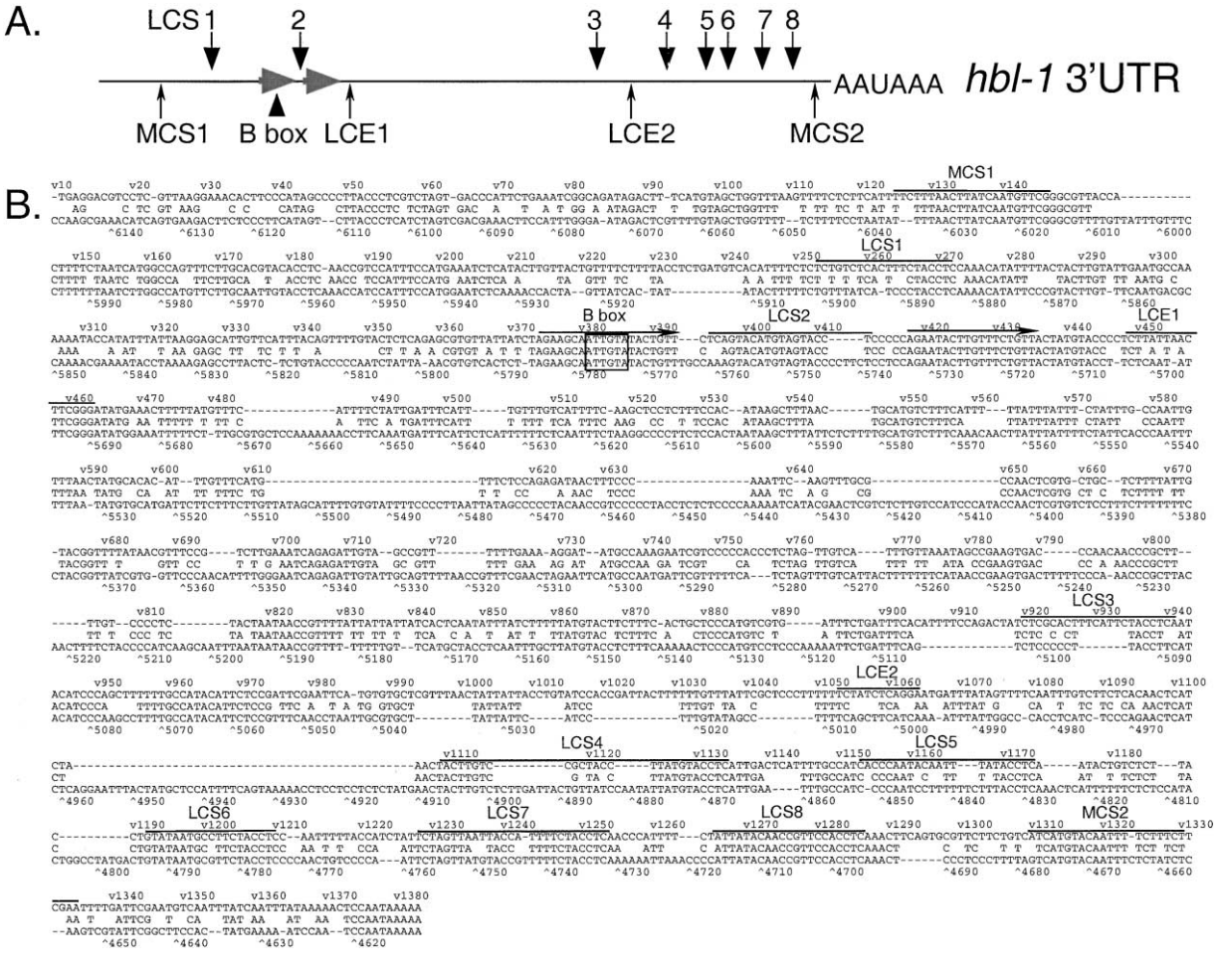

Figure 4. The *hbl-1* 3′UTR

(A) Diagram of the *hbl-1* 3′UTR showing potential binding sites for the miRNAs (*let-7* complementary sites [LCSs] and those of its homologs, *lin-4* complementary elements [LCEs], and *mir-69* complementary sites [MCSs]). Horizontal arrows represent a direct repeat containing potential divergent *nanos* response elements.

(B) Alignment of the *hbl-1* 3′UTR from *C. elegans* (top) and *C. briggsae* (bottom) showing conserved regions. Lines above the alignment indicate the positions of miRNA binding sites, which are numbered to correspond to (A). The direct repeats with potential divergent NREs are indicated by arrows. The canonical B box is boxed. Numbering for *C. elegans* is from the start of the *hbl-1* 3′UTR, and the numbering for *C. briggsae* is from c006800689.Contig1 (Jim Mullikin's PHUSION assembler data [1/15/02] [the Sanger Institute and the Genome Sequencing Center, Washington University, St. Louis]).

et al., 2001; Lee and Ambros, 2001; Mourelatos et al., 2002) and plants (Reinhart et al., 2002). Many of these are evolutionarily conserved, suggesting that they perform fundamental roles in the organism. The two best understood miRNAs, *lin-4* and *let-7*, function in the heterochronic pathway to posttranscriptionally regulate expression of their target genes in a 3′UTR-dependent manner. To date, relatively few miRNAs targets have been identified. Here we demonstrate that *hbl-1*, the *C. elegans* ortholog of *Drosophila hunchback*, is a heterochronic gene and is likely to be controlled by at least two miRNAs, *let-7* and *lin-4*, through its 3′UTR.

hbl-1 Controls Temporal Identity of Seam Cells

We reinvestigated the postembryonic role of *hbl-1* and found that it controls temporal patterning. Knockdown of *hbl-1* activity leads to precocious expression of adult hypodermal fates in the L4. Thus, *hbl-1* acts before the L4 stage to prevent the precocious expression of adult fates at the L4 stage. Adult fates in the hypodermis are specified by *lin-29* (Ambros and Horvitz, 1984; Rougvie and Ambros, 1995). Reduction in *hbl-1* function fails to cause a precocious phenotype in a *lin-29* retarded mutant background; therefore, the *hbl-1* repression of adult fates during the larval stages likely involves the negative regulation of *lin-29* expression or function. *lin-29* therefore acts downstream of, or in parallel to, *hbl-1*. Interestingly, *lin-29* encodes a Krüppel family transcription factor (Rougvie and Ambros, 1995), which is a downstream Hunchback target in *Drosophila* (Struhl et al., 1992).

Our genetic data suggests that *lin-41* and *hbl-1* act together or in parallel pathways to repress inappropriate, precocious LIN-29 activity during late larval stages (Figure 6). Future work will determine whether the *hbl-1-lin-41* interaction is direct or indirect.

hbl-1 appears to play both negative and positive roles in regulating adult fates in the hypodermis. *hbl-1* may

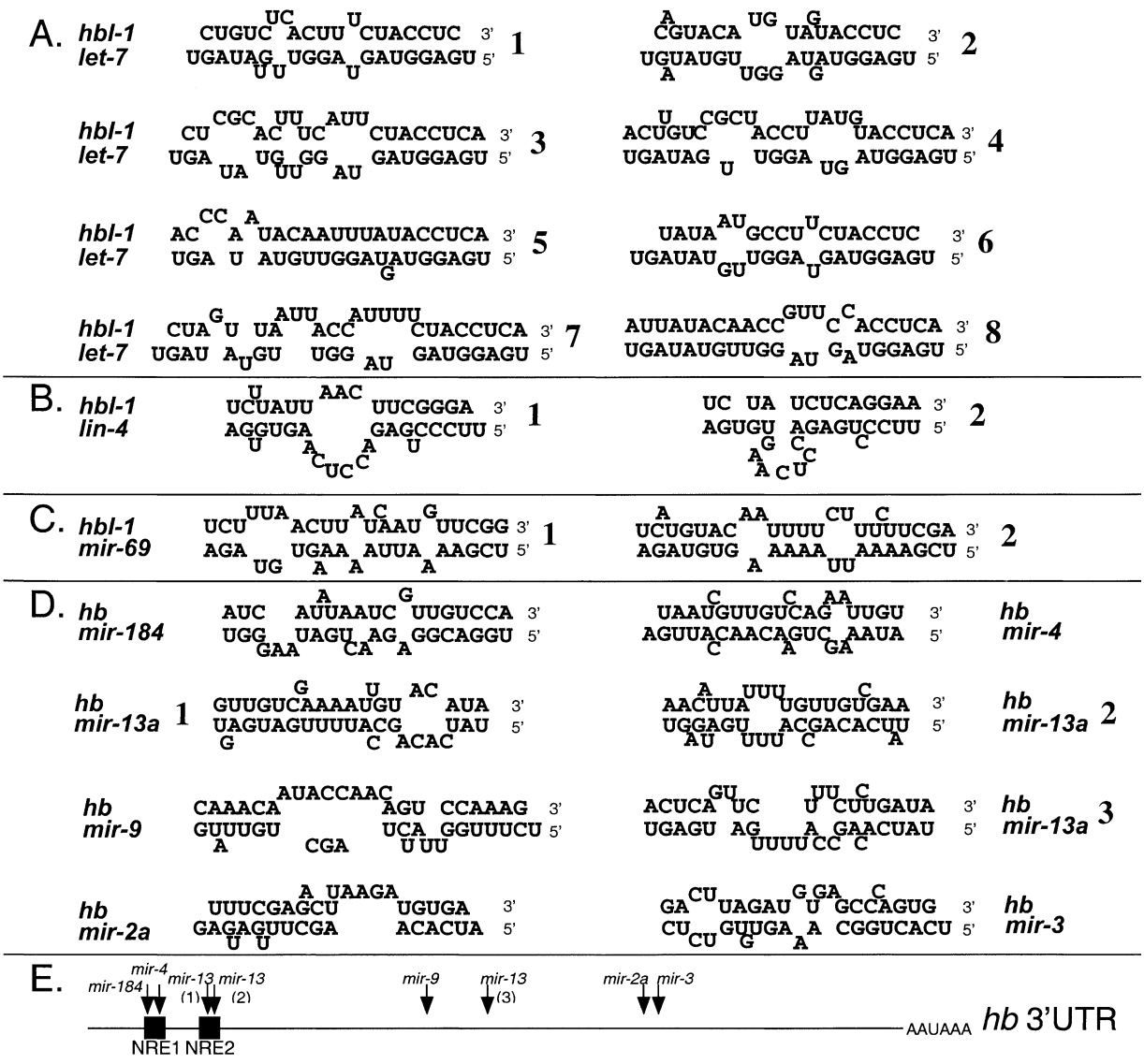

Figure 5. Interactions between the miRNAs and the *hbl-1*/*hb* 3′UTR RNA

Potential RNA::RNA duplexes between *hbl-1* and *let-7* (A), between *hbl-1* and *lin-4* (B), and between *hbl-1* and *mir-69* (C), as identified by a combination of manual searching and computer analysis with the mfold program (Mathews et al., 1999). The *hbl-1* 3′UTR is predicted to contain at least eight LCSs, two LCEs, and two MCSs, numbered to correspond to Figure 4A. While duplexes are shown with *let-7* RNA, any of the LCS sequences could potentially form duplexes with any of the *let-7* homologs. Thermodynamically more-stable duplexes can be formed between LCS1 and either *let-7* RNA or *mir-48* RNA, between LCS2 and *let-7* RNA, between LCS3 and *mir-84* RNA, between LCS4, 5, 6, and 7 and either *let-7* RNA or *mir-84* RNA, and between LCS8 and *let-7* RNA.

(D) Potential RNA::RNA duplexes between *Drosophila hb* and various indicated *Drosophila* miRNAs.

(E) Diagram of the *D. melanogaster hb* 3′UTR. The black boxes represent NREs. Arrows mark the sites of sequences complementary to the indicated *Drosophila* miRNAs.

also promote or maintain adult fates, since loss of *hbl-1* function appears to cause some adult hypodermal nuclei to reiterate their larval fates and divide. The interpretation of this result is complicated because the seam cells have precociously fused to form the seam syncitial cell before the adult stage, so we observe reiteration of nuclear division, but not cell division. Conceivably, HBL-1 negatively regulates genes required for the L/A switch in early larval stages and positively regulates genes required to promote or maintain adult fates in the L4 or adult stage. Since HBL-1 concentrations decrease over developmental time, HBL-1 could function differently at different concentrations. As part of its essential role in anterior-posterior axis specification during *Drosophila* embryogenesis, Hunchback acts along a concentration gradient both as a negative and positive transcriptional regulator of gene expression (Struhl et al., 1992). Hb represses *Kr* expression at high concentrations, but activates it at low concentrations (Schulz and Tautz, 1994). Conceivably, *hbl-1* could repress *lin-29*

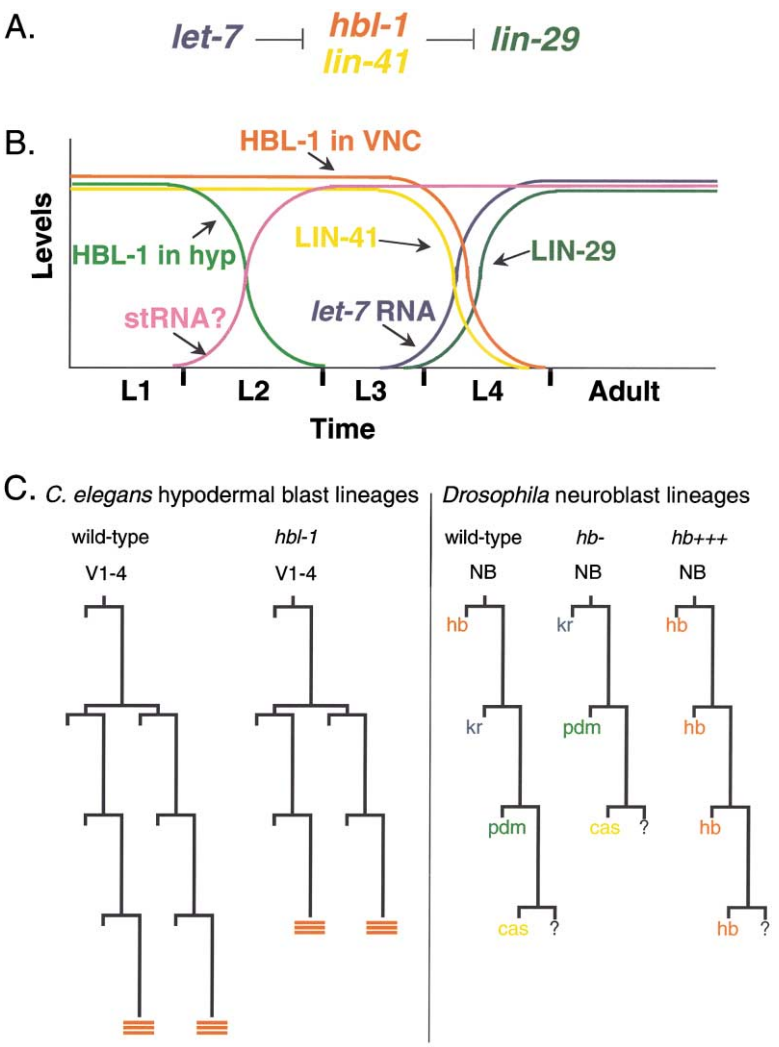

Figure 6. Model of Heterochronic Gene Activities

(A) Genetic data support a model whereby the heterochronic gene *let-7* negatively regulates *hbl-l. hbl-1*, in turn, negatively regulates *lin-29*, possibly with *lin-41*. The simplest interpretation of the hypodermal data is consistent with the genetic pathway, as shown.

(B) Heterochronic gene expression over time in ectodermal tissues. Expression of *hbl-1* (in neurons; red) and *lin-41* (in hypodermis; yellow) both appear to be posttranscriptionally regulated by the appearance of *let-7* RNA (blue). HBL-1 expression in the non-seam cell hypodermis (light green) is likely to be regulated by other stRNAs (purple).

(C) Similarities between *hbl-1* mutant defects in seam cell lineages and *hb* mutant defects in *Drosophila* neuroblast lineages. The *C. elegans* cell lineages of the lateral hypodermal V1–4 cells in wild-type and *hbl-1(mg285)* (proposed) animals are shown. The vertical axis indicates the four postembryonic larval stages divided by molts. In wild-type animals, the V1–4 cells divide close to the beginning of each larval stage until the final molt, when they terminally differentiate and secrete adult specific cuticular alae, indicated by the triple bars.

at high concentrations (i.e., at early larval stages) and activate *lin-29* at low concentrations (i.e., during late larval and adult stages).

hbl-1 Is Temporally Downregulated during the Course of Postembryonic Development

We and others (Fay et al., 1999) have observed that *hbl-1* is temporally downregulated in hypodermal and neuronal cells. HBL-1/GFP expression levels are highest in embryos, become progressively less abundant through the larval stages, and are minimal in adults. This is consistent with developmental Northern blots, where *hbl-1* mRNA levels are high in embryos, reduced in L1 and L2 animals, and barely detectable by the L3 stage (data not shown; Fay et al., 1999).

hbl-1 Is Downregulated through 3′UTR-Dependent and -Independent Mechanisms

HBL-1/GFP is initially observed in hypodermal seam cell nuclei at hatching but is not detected in seam cell nuclei at the end of the L1 stage. This downregulation is 3′UTR independent, since HBL-1/GFP expressed from a construct containing the *unc-54* 3′UTR is absent from seam cells at the end of the L1 stage. Thus, we believe that *hbl-1* downregulation in the seam cells during the L1 stage is due to transcriptional control of *hbl-1*.

We observed that HBL-1/GFP is downregulated by a second mechanism that requires the *hbl-1* 3′UTR and is therefore expected to occur posttranscriptionally. The *hbl-1* 3′UTR is necessary for HBL-1/GFP downregulation in both the non-seam cell hypodermis (by the L3 stage) and the VNC (by the adult stage), since the HBL-1/GFP-*unc54* 3′UTR construct was not downregulated over time, with high HBL-1/GFP expression in adult tissues. The *hbl-1* 3′UTR is also sufficient to downregulate a heterologous reporter gene driven by a nonregulated promoter in the hypodermis. Thus, two lines of evidence highlight the existence of a 3′UTR-dependent mechanism in the hypodermis.

Fay et al. (1999) first demonstrated that *hbl-1* mRNA, present in L1 animals, is absent in later larval and adult animals, a result that we confirmed. Since the *hbl-1* promoter is active in late larval and adult stages (this work) and the *hbl-1* mRNA is not observed concurrently, this suggests that the 3′UTR-mediated, posttranscriptional downregulation of HBL-1 reflects altered *hbl-1* mRNA stability over time. Three other heterochronic

genes, *lin-14, lin-28*, and *lin-41*, are known to be regulated through their 3′UTRs. However, the stability of these RNAs is not altered over time (Wightman et al., 1993; Moss et al., 1997; M.C.V. and F.J.S., in preparation). Future work will reveal whether miRNAs employ different mechanisms of posttranscriptional control depending on the target gene.

The *hbl-1* 3′UTR Contains Multiple miRNA Complementary Sites

miRNAs are predicted to bind complementary sequences in the 3′UTR of their targets and downregulate them posttranscriptionally. *lin-4* complementary elements (LCEs) and *let-7* complementary sites (LCSs) exist in the 3′UTRs of *lin-14*, *lin-28*, and *lin-41*. Examination of the *hbl-1* 3′UTR identified two LCE and eight LCS elements, each with the potential to form RNA/RNA duplexes with the corresponding miRNA. Recently, multiple *C. elegans* miRNAs have been identified (Lau et al., 2001; Lee and Ambros, 2001), including two (*mir-48* and *mir-84*) with sequence similarity to *let-7*. These *let-7* homologs, like *let-7* and *mir-69* RNA, are temporally expressed at similar times in late larval stages (Lau et al., 2001). Since the homologs are so similar in sequence to *let-7* RNA, it is difficult to definitively assign an LCS sequence because they might bind one or more of the *let-7* homologs. The other temporally expressed miRNA, *mir-69*, is also predicted to bind to evolutionarily conserved *hbl-1* 3′UTR sequences. Thus, the *hbl-1* 3′UTR has the potential to be regulated by multiple miRNAs. This raises the enticing possibility that posttranscriptional regulation may be mediated by the antagonistic, competing, and/or cooperative binding of miRNAs to sites in their target 3′UTRs, similar to modular control of transcription mediated by transcription factors binding to promoters.

hbl-1 Is a Probable Downstream Target of the *let-7* miRNA

Multiple lines of evidence suggest that *hbl-1* is downstream of *let-7*. We failed to observe an effect of *hbl-1* loss-of-function on the *let-7* RNA temporal expression pattern relative to wild-type. Also, *hbl-1* activity or expression appears to be upregulated in a *let-7* mutant, since loss of *hbl-1* function strongly suppresses the lethal effects and partially suppresses the heterochronic defects associated with a *let-7* mutant. Moreover, the presence of conserved *let-7* complementary sites in the *hbl-1* 3′UTR strongly suggests that *hbl-1* is a direct downstream target of *let-7* RNA. Consistent with this interpretation, *let-7* RNA binds *hbl-1* mRNA 3′UTR in vitro (E.-Y. Choi and F.J.S., unpublished data). Although we do not yet have evidence for a direct interaction in vivo, we observed that HBL-1/GFP is downregulated in the VNC, coincident with *let-7* RNA expression. Furthermore, this temporal downregulation is *let-7* dependent because it is altered in *let-7* mutants. These data together demonstrate that *let-7* is at least partially responsible for downregulating neuronal *hbl-1* expression. *lin-4* also seems to function in this process, since temporal downregulation in VNC neurons completely fails in a *lin-4* mutant. This interaction could occur directly, via the *hbl-1* 3′UTR LCE sites.

let-7 May Function Differently at Different 3′UTRs

let-7 RNA functions in neuronal, but not in hypodermal, *hbl-1* downregulation. Interestingly, *lin-41*, which has multiple LCS sequences in its 3′UTR, is downregulated in the hypodermis, but not in neurons (Slack et al., 2000). Also, unlike the case with *hbl-1* 3′UTR, *let-7* is required for the downregulation of a hypodermally expressed *lacZ-lin-41* 3′UTR reporter (Reinhart et al., 2000; Slack et al., 2000). Thus, *lin-41* appears to be a *let-7* target in the hypodermis, but not in neurons, while the opposite seems true for *hbl-1*. Therefore, the predicted *let-7* complementary sites in the *lin-41* and *hbl-1* 3′UTRs differ in the nature of their *let-7* regulation. Perhaps the spatial context of mRNA localization, cofactors, or 3′UTR architecture determines whether, to what extent, and by which mechanism *let-7* regulates target gene expression via the 3′UTR.

Conservation of a Temporal Role for *hunchback* in Evolution

hb is best known for its essential role in spatial patterning in the *Drosophila* embryonic anterior (Irish et al., 1989) and was recently shown to regulate temporal neuroblast identity (Isshiki et al., 2001). Neuroblasts divide with a stem cell-like lineage to generate four classes of ganglion mother cells (GMCs), derived from four sequential neuroblast divisions (Figure 6C). The first-born GMCs express *hb*, and the second-born GMCs express *Kr*. In *hb* and *Kr* mutants, precocious expression of later GMC fates is observed. In contrast, *hb* overexpression causes reiteration of first-born fates (Figure 6C). In *C. elegans*, we have similarly observed precocious expression of later fates in *hbl-1* mutants. *hb* homologs have now been shown to control temporal patterning in different phyla, underscoring a potential evolutionarily conserved role for *hb* family members in controlling temporal identity.

As illustrated by *Drosophila hb* and *C. elegans hbl-1*, spatial and temporal patterning may share certain features. Functions of key genes and their mechanisms of regulated expression are conserved. In *Drosophila* spatial patterning and *C. elegans* temporal patterning, *hb* and *hbl-1* expression are both regulated transcriptionally and translationally. Within the *Drosophila* embryo posterior, *hb* expression is repressed posttranscriptionally via Nanos response elements (NREs) in the *hb* 3′UTR (Murata and Wharton, 1995; Sonoda and Wharton, 1999; Wharton et al., 1998). NRE sequences are bipartite 3′UTR elements consisting of an A box (GUUGU), a spacer, and a B box (AUUGUA) (Barker et al., 1992; Tautz and Pfeifle, 1989; Wharton and Struhl, 1991). While the A box and spacer can exhibit variability, the B box is highly conserved (Dalby and Glover, 1993; Gamberi et al., 2002; Murata and Wharton, 1995). Functional control is mediated through B box recognition by the prototypical Puf protein Pumilio (Murata and Wharton, 1995) and recruitment of additional factors, including Nanos and Brat in the case of *hb* (Sonoda and Wharton, 1999, 2001). We have identified two sequences in the 3′UTR of *hbl-1* that may define modified NREs (Figure 4B, arrows), both of which are 100% conserved in *C. briggsae*. The most proximal element contains a canonical NRE B box and an upstream region with A

box similarity (Figures 4A and 4B). The distal potential site contains a divergent B box. The *C. elegans* genome encodes 11 Pumilio (FBFs and PUFs) and three Nanos (NOS) homologs (Wickens et al., 2002). Conceivably, a subset of these homologs can recognize these NRE-like sequences. Indeed, the *hbl-1* B box contains the UGUR core sequence required for PUF protein recognition, and other potential core sequences reside throughout the 3′UTR. Intriguingly, the *hbl-1* B box clusters with predicted *let-7* and *lin-4* RNA binding elements (Figure 4B), raising the possibility that these miRNAs may interact with B box binding proteins.

Given the similarities in *hb* and *hbl-1* regulation, we speculate that miRNAs may control *hb* expression in *Drosophila*. While we did not find *let-7* complementary sites in the *Drosophila hb* 3′UTR, other miRNAs might provide this function. Notably, we identified sites complementary to eight different *Drosophila* miRNAs in the *hb* 3′UTR (Figures 5D and 5E). Four of these, complementary to *mir-4, mir-13* (twice), and *mir-184*, reside in regions highly conserved between different *Drosophila* species, and they overlap NRE elements (Figure 5E).

It is currently unclear whether *hb* temporal expression in developing neuroblasts reflects transcriptional and/or posttranscriptional regulation. Since our work with *C. elegans hbl-1* suggests extensive conservation of genetic factors, it would not be surprising if *hb* is post-transcriptionally regulated in the neuroblasts via miRNA action.

Finally, our data, together with that of Issiki et al. (2001), raise the question of what distinguishes the well-characterized embryonic spatial roles and the newly characterized temporal roles for *hunchback* homologs and to what extent the genetic interactions between the two roles are conserved. If spatial and temporal programs are distinct, *hunchback* function could have originally evolved for spatial patterning but was subsequently sequestered by temporal patterning programs (or vice versa). Alternately, because spatial patterns manifest themselves during development (i.e., along the temporal axis), it is often difficult to dissociate temporal versus spatial patterning. For example, recent work suggests that temporal alterations in Bicoid expression during *Drosophila* embryogenesis result in altered spatial patterns (Gamberi et al., 2002). Spatial and temporal patterning programs could be mechanistically very similar and may even be equivalent.

Experimental Procedures

Screen for Heterochronic Genes

We mutagenized N2 hermaphrodites with ethylmethane sulfonate and screened for mutants displaying egg-laying (Egl), protruding vulval (Pvl), and cuticle defects characteristic of known heterochronic mutants. From 540,000 mutagenized genomes scored in the F1 generation, one mutant we obtained, *mg285*, met the criteria for a precocious mutant. We found that *mg285* is recessive: recessive mutations can arise in an F1 screen if some F2 progeny are accidentally scored.

Mapping of *mg285*

mg285 was mapped near *lon-2* on LGX by standard genetic tests. From *lon-2/mg285* animals, 4 out of 76 *mg285* recombinants segregated Lon-2 animals. This result suggests that *mg285* maps within 2.5 map units of *lon-2*. Sequencing the *hbl-1* gene from *mg285* mutants confirmed that *mg285* is an allele of *hbl-1*.

Phenotypic Analysis

Cell anatomical analysis was performed as described (Ambros and Horvitz, 1984; Sulston and Horvitz, 1977). To visualize seam cell junctions and nuclei, we utilized an integrated array (*wIs79*) containing both *ajm-1::gfp* (MH27/GFP) and *scm-1::gfp*. To visualize HBL-1/GFP expression, we used strains BW1891 and BW1932, containing integrated *hbl-1::gfp* fusions (Fay et al., 1999).

Transgenics and Northerns

A *col-10-lacZ-hbl-1* 3′UTR construct was created by amplifying the *hbl-1* 3′UTR from an *hbl-1* cDNA clone, yk421f4, with the primers F13D11(16) and F13D11(17) (sequences available on request) and then digesting it with SacII and NcoI. This product was subcloned into plasmid B29 (Wightman et al., 1993) digested with SacII and NcoI to create pFS1038. Transgenic animals were created by coinjecting pFS1038 at 5 ng/μl and pKP13 (*goa-1::gfp*) at 50 ng/μl. Seven independent lines, carrying extrachromosomal arrays, were tested for β-galactosidase expression. Results from all lines showed the same trend and were averaged. The developmental Northern was performed as described (Reinhart et al., 2000).

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